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(54) Title: RECOMBINANT RHIZOBIUM BACTERIA INOCULANTS

(57) Abstract

The sequences of a *Rhizobium* bacteria responsible for competitiveness with respect to plant nodulation have been isolated and permanently transferred to superior nodulating *Rhizobium* genome. This has resulted in a stable construct that can form a plant inoculant that yields effective nodulation, while reducing the risk of suppression by other bacteria in the environment.

* See back of page

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RECOMBINANT RHIZOBIUM BACTERIA INOCULANTS

Background Of The Invention

Technical Field

The present invention relates to recombinant DNA technology. It appears especially useful for improving the nodulation (and thus nitrogen fixation) capability of plants.

Background Art

Root nodule Rhizobium bacteria are responsible for symbiotic nitrogen fixation in the nodules of certain plants (e.g. legumes). Where natural bacterial activity is ineffective, the plants must rely on the existing nitrogen in the soil or on fertilizers. Where the former occurs, the quality of the soil is reduced. Where the latter occurs, the cost to the farmer (and ultimately the public) can be substantial. Further, the use of fertilizers often raises environmental concerns.

It is now known that the presence of certain "inferior" strains of Rhizobium in soil can depress the productivity of not only other natural bacteria, but also of "superior" bacteria added by inoculation of seeds. This can frustrate attempts to inoculate seeds prior to planting or to inoculate roots during plant growth. When inoculation has been successful, it is usually because the indigenous bacterial populations have been small.

Many investigators have studied the factors involved in determining nodule occupancy by strains of Rhizobium. See e.g. D. Dowling et al., 40 Annu. Rev. Microbiol. 131-157 (1986) (the disclosure of this article and of all other articles referred to herein are incorporated by reference as if fully set forth). Despite this work, no solutions to the above described Rhizobium competition problem have been developed.

In E. Triplett et al., 85 Plant Physiology 335-342 (1987) and 11th North American Rhizobium Conference Abstract GP4 (1987), my laboratory reported on the fact that the Rhizobium leguminosarum bv. trifolii bacterial strain T24 appeared to have genes in its coding responsible for a suppressor of other Rhizobium (I named the substance trifolitoxin) and other genes coding for T24's own resistance to trifolitoxin's effects. Unfortunately, I also have found that trifolitoxin production by transconjugant bacterial cells that I had constructed was readily lost in the absence of tetracycline. Thus, the earlier Rhizobium transconjugants were not likely to be able to effectively limit nodulation by trifolitoxin-sensitive indigenous strains of Rhizobium under agricultural conditions (where tetracycline application is impractical).

It was therefore desired to more specifically isolate and characterize the genes responsible for the T24 suppressor and resistance characteristics and use information developed therefrom to find a means for stably inserting such genes in the genome of "superior" Rhizobium so as to ultimately lead to a Rhizobium that can form effective nodules notwithstanding the presence of indigenous strains.

Disclosure Of Invention

The invention provides a recombinant Rhizobium bacteria capable of assisting in the formation of nitrogen fixation nodules on at least some plants. The bacteria has a foreign sequence expressibly coding for trifolitoxin. The bacteria preferably also has a sequence coding for resistance to trifolitoxin suppression, with both foreign sequences being in the bacterial genome. The term "genome" is used herein to refer to either the bacterial chromosome or other bacterial genetic sequences in the bacteria.

Inoculants can be provided that use these bacteria. Thus, plant seeds (or the roots of young plants) can b inoculated with the bacteria.

Further, plant cells can be formed that incorporate these sequences (so that the plant strain produces its own trifolitoxin). In the alternative, a production host can produce trifolitoxin on a commercial scale. In either case, the trifolitoxin can be used as a trans inoculant by having the superior strain have the resistance gene only.

It will be appreciated that the invention provide the ability to effectively create nitrogen fixation nodules in the presence of inferior strains.

The objects of the invention therefore include:

A. providing a recombinant bacteria of the above kind;

B. providing a recombinant host of the above kind;

C. providing a plant seed inoculated with a bacteria of the above kind; and

D. providing a plant inoculant using a bacteria of the above kind.

These and still other objects and the advantages of the present invention will be apparent from the description that follows.

Best Modes For Carrying Out The Invention

General Overview

Rhizobium leguminosarum bv. trifolii T24 induces ineffective nodules but produces a potent anti-rhizobial compound, trifolitoxin. As a result of trifolitoxin production, T24 prevents root nodulation by trifolitoxin-sensitive bacterial strains. The main objective of this work was to identify and isolate the trifolitoxin production and resistance genes and permanently transfer those gene to other strains of Rhizobium that produced "superior" nodules.

To achieve this, a genomic library of T24 was prepared in the prior art cosmid vector pLAFR3. One cosmid clone was identified that restored trifolitoxin production and nodulation competitiveness. We formed a recombinant plasmid from this cosmid clone, pTFX1, that conferred trifolitoxin production and resistance on other bacteria (albeit in an unstable fashion).

Transposon mutagenesis and restriction analysis was then used to map and subclone the insert of pTFX1. A 4.4 kb region of DNA, referred to as tfx was found to be necessary for the expression of trifolitoxin production and resistance in Rhizobium. Another portion was found to have sufficient homology to Rhizobium genome to permit the use of a technique for insertion into the genome. Several mutants of pTFX1 (with Tn5 insertions outside the trifolitoxin region) were therefore used to permanently insert the trifolitoxin genes into several strains of Rhizobium. This resulted in a stable construct having the desired characteristics.

Methods And Materials

The identification of the precursor cosmid clone, and the formation of plasmid pTFX1 is described in detail in my article, E. Triplett, 85 P.N.A.S. USA 3810-3814 (June 1988) (not prior art). I then made a restriction map of pTFX1. I did this by restriction analysis of Tn5 insertions in pTFX1. This map was used to determine the size and location of the trifolitoxin genes as well as to develop a strategy to subclone the trifolitoxin genes into the broad host range vector, pRK415, N. Keen et al., 70 Gene 191-197 (1988). I found that the ability of pTFX1 to confer trifolitoxin production as well as resistance in trifolitoxin-sensitive strains of Rhizobium were located within a 4.4 kb region of pTFX1, this knowledge, plus my analysis of the other portions of the insert in turn led to selection of the marker exchange technique for inserting these genes in a bacterial genome.

In my work, Rhizobium strains were cultured at 28 C on Bergersen's synthetic medium (BSM) as described by F. Bergersen, 14 Aust. J. Biol. Sci. 349-360 (1961). Strains of E. coli were cultured at 37°C on Luria-Bertani (LB) medium. Antibiotics were added as needed at the following final concentrations: kanamycin (Km), 50 ug/ml; tetracycline (Tc), 12.5 ug/ml; spectinomycin (Sp), 50 ug/ml; streptomycin (Sm), 50 ug/ml; gentamycin (Gm), 25 ug/ml; nalidixic acid (Nal), 10 ug/ml; and neomycin (Nm), 75 ug/ml.

Conjugation of the plasmid mutants (e.g. pTFX1::Tn5) into Rhizobium was performed using procedures analogous to those described in E. Triplett et al., 85 Plant Physiol. 335-342 (1987) with some modifications. In this regard, the donor, recipient, and helper strains were mixed in a 1:1:1 ratio in water each at a cell density of approximately 5 x 10^7 per ml. After vortexing, a 5 ul suspension of this mixture is plac d on a YM/KB (se E.

Triplett (1987), supra) plate with 3% agar. After incubation for two days at 28° C, each mating was resuspended in 0.1 ml water and spread plate on a BSM plate prepared with noble agar and supplemented with tetracycline and streptomycin. The use of noble agar in the interruption media eliminated the background of growth on the plates. After five days, transconjugants were observed.

Conjugations involving the transfer of plasmid DNA between strains of E. coli were done as described above except that 5 ul of the mixture of donor, recipient, and helper strains were placed on an LB plate and incubat d at 37°C overnight. Interruptions were done as describ d above with the appropriate selective media on solid LB medium.

In the transfer of plasmid DNA from E. coli to Rhizobium, E. coli DH5a (Bethesda Research Labs) (pRK2013), D. Figurski et al., 76 P.N.A.S. USA 1648-1652 (1979), was used as the helper strain. In the transfer of plasmid DNA between two strains of E. coli, E. coli HB101, H. Boyer et al., 41 J. Mol. Biol. 459-472 (1969), (pRK2073) (S. Leong et al., 257 J. Biol. Chem. 8724-8730 (1982)) served as the helper strain.

Large scale plasmid preparations were purified by the boiling method described by D. Holmes et al., 114 Anal. Biochem. 193-197 (1981). For restriction analysis of small amounts of plasmid DNA, plasmids were purified from cells grown on sold medium by the alkaline lysis miniprep method described by F. Ausubel et al., Current Protecols In Molecular Biology (1987).

The recombinant plasmid, pTFX1, was mutagenized with Tn5 by the method of G. Ditta, 118 Meth. Enzmol. 519-528 (1986) with slight modifications. The plasmid pTFX1 was transformed into E. coli cell line HB101::Tn5 as described by D. Hanahan, 166 J. Mol. Biol. 557-580 (1983) using LB medium supplemented with kanamycin and tetracycline for selection of transformants. The transformants

were pooled and conjugated with HB101 (pRK2073) and C2110nal (Ditta, supra). The triparental matings were incubated overnight at 37°C. Cells were resuspended in water and a dilution series plated on LB medium supplemented with kanamycin, tetracycline, and nalidixic acid. Transconjugants were pooled and plasmid DNA isolated by an alkaline lysis miniprep procedure as described by F. Ausubel et al., Current Protocols In Molecular Biology, John Wiley & Sons, New York (1987). Fourteen separate matings were performed in order to enhance the prospects of obtaining independent mutations. Plasmid DNA was transformed into E. coli DH5a and the subsequent transformants selected on LB medium with kanamycin and tetracycline.

Restriction Analysis

Restriction analysis of three hundred and thirty-six pTFX1::Tn5 mutants was done to provide the information necessary to construct a restriction map of pTFX1. Each mutant was also conjugated into R. leguminosarum bv. trifolii strain TA1 as described above to determine the trifolitoxin phenotype. (The tfx genes are not expressed in E. coli.)

Plasmid DNA of each pTFX1::Tn5 mutant was cleaved with the following restriction enzymes: Eco RI, Kpn I, Dra I, and Mlu I. To accurately map Tn5 insertions within each restriction fragment, selected pTFX1::Tn5 plasmids were cleaved with Hpa I, an enzyme with two symmetrical restriction sites within the inverted repeat sequence elements of Tn5. Hpa I has two restriction sites in pTFX1. This enzyme was used for this purpose rather than Bgl II since there are no Bgl II restriction sites in pTFX1. Plasmid DNA was electrophoresed in 0.6% agarose at 100 v. For the separation of fragment sizes greater than 15 kb, field inversion electophoresis was

used. At 0.3 s intervals, the electric field was inverted between 100 v toward the anode and 60 v toward the cathode. Field inversion gels were run for 16 hours at 4°C. All gels were 10 cm in length.

The ability of a strain to produce trifolitoxin was determined by bioassay; Southern analysis was determined with biotinylated probes of either pLAFR3 or pTFX1; and trifolitoxin was partially purified from the cell culture supernatants of T24 and various Rhizobium transconjugants containing pTFX2 by reverse phase chromatography; these steps all being done as described in the general technique portion of E. Triplett, 85 P.N.A.S. USA 3810-3814 (June 1988) (not prior art).

From this analysis, I determined that my previous estimate of the size of the insert in pTFX1, 24.2 kb was inaccurate. The insert size in pTFX1 is now known to be 29.5 kb.

The restriction analysis showed that two enzymes, Dra I and Mlu I, did not have restriction sites in either Tn5 or tfx. The tfx region resides on a 10 kb Dra I fragment and a 7.5 kb Mlu I fragment. Since tfx is present on a smaller fragment in the Mlu I digest than in the Dra I digest, Mlu I fragments were chosen for subcloning tfx.

Subcloning

One mutant of pTFX1 was chosen whose Tn5 insertion was located within the 7.5 kb Mlu I fragment of pTFX1, yet did not affect the expression of trifolitoxin production in Rhizobium. Ligation of the Mlu I fragments from which contains both the intact trifolitoxin production genes and a Tn5 insertion on the same fragment, to the broad host-range vector, pRK415, allows for selection against the other possible ligation products.

An Mlu I digest of plasmid DNA from a pTFX1::Tn5 mutant was blunted with T4 DNA polymerase using techniques described by F. Ausubel et al., Current Protocols In Molecular Biology, John Wiley & Sons, New York (1987). These fragments were ligated to an alkaline phosphatase-treated Xmn I digest of pRK415. The resulting ligated DNA was transformed into DH5a competent cells and transformants selected on LB solid medium supplemented with kanamycin and tetracycline. Restriction analysis of the plasmid DNA of a selected transformant showed an insert size of 13.2 kb (as was predicted based on the size of an Mlu I fragment of pTFX1 with a Tn5 insertion). This plasmid is referred to as pTFX2.

A restriction map of pTFX2 was prepared based on the restriction sites known to be present in pRK415, the Eco RI restriction sites present in the Mlu I fragment in pTFX1, and on double restriction digests of pTFX2 with Sst I, Eco RI, and Hpa I. The Xmn I site in pRK415 and the Mlu I sites in the insert were eliminated by the blunt end ligation of the insert into the vector.

To determine whether pTFX2 possessed functional tfx, this plasmid was conjugated into Rhizobium. Trifolitoxin production was observed by the resulting transconjugants and confirmed using techniques described previously for pTFX1 transconjugants in E. Triplett 1988, supra (not prior art).

Insertion Into Bacterial Genome

As an example of inserting tfx into a selected bacterial genome, the method of G. Ditta, 118 Meth. Enzmol. 519-528 (1986) was adapted for R. leguminosarum by. trifolii TA1. (A. Gibson, CSIRO) The technique starts from the idea that certain plasmids may be incompatible with certain other plasmids in certain hosts, and that under antibiotic stress the host will tend to either

drive one out (or hopefully where homology exists take in the unwanted genetic material as part of the bacterial genome). The incompatible plasmid pPH1JI (J. Bering r, 276 Nature 633-634 (1978)) was conjugated into several TA1 transconjugants with my pTFX1::Tn5. It will be appreciated that the host Rhizobium can be other "superior" hosts of interest. The conjugation was int r-rupted on BSM prepared in noble agar and supplemented gentamycin, kanamycin, and spectinomycin. The resulting exconjugants (with the gene in the cell genome) were replica-plated on BSM with tetracycline. The tetra-cycline-resistant strains were discarded.

Bacterial strains T24, TA1 (pTFX1), and trifolitoxin-producing TA1 (pTFX1::Tn5) transconjugants and TA1::TFX::Tn5 exconjugants were streaked to single colonies on BSM medium in the absence of selective anti-biotics. After two days of incubation at 28°C, a portion of the confluent growth on the plate was suspended in water and 5 ul of that suspension spotted in the center of a BSM plate for the assay of trifolitoxin production. A single colony from the initial plate was used to inocu-late a second plate. After two days, confluent growth on the second plate was used to assay trifolitoxin. The assays continued for 10 "generations" or until trifoli-toxin production was no longer observed. TA1::TFX:Tn5 showed stability through ten generations.

It will be appreciated that the present invention involves, inter alia, the location of the trifolitoxin production and resistant genes, the cloning of them, and the development of a way to insert them permanently in the bacterial genome.

Cultures of pTFX1::Tn5 (a/k/a pTFX1:10-15) in E. coli (DH5 alpha) and Rhizobium leguminosarum bv. trifolii TA1::TFX:Tn5 (a/k/a TA1::10-15) are on deposit at the American Type Culture Collection, Rockville, Maryland, U.S.A., with ATCC numbers 67990 and 53912 respectiv ly.

They were deposited May 25, 1989 under the Budapest Treaty. They will be made available upon issuance of this patent and as provided under U.S. and other applicable patent laws. However, this availability is not to be construed as a license to use the invention.

The preferred way to use the preferred bacteria is to streak the deposited TA1 :10-15 on BSM solid AGAR and wait for 2-3 days. One then streaks the growth product into BSM liquid broth. After several more days one can pour the liquid broth on peat and uses the peat as a carrier to surround the seeds or roots. Note also that other known commercial inoculant techniques can readily be adapted for use with these bacteria. See e.g. R. Roughley et al. in Nitrogen Fixation In Legumes, p193-209 (1982); resulting in inoculants and inoculated seeds. This invention appears most likely to be useful on clover, peas, beans, vetch, and soybeans, but may well have utility wherever Rhizobium created nodules.

Another possible use of the invention is to insert only the resistance gene in a bacteria and then add trifolitoxin to the soil (or transform a plant cell so it produces the trifolitoxin). In this regard, several vectors are already known that can expressibly transform a plant genome, and many commercial production hosts are known.

Other Variants

It will be appreciated that various other changes to the preferred embodiment may be made. For example, various other strains besides T24 may produce trifolitoxin, and thus their sequences could be used (e.g. after location with a hybridization probe based on pTFX1). Also, means of inoculating the roots of live plants (as opposed to just seeds) during transplantation can easily be developed using known techniques. Further, other

means for inserting the foreign genes in the bacterial chromosome may prove useful. See e.g. G. Barry, 4 Bio/Technology 446-449 (1986) and 71 Gene 75-84 (1988). Th claims should therefore be looked to to judge the full scope of the invention and the preferred embodiment is not to be considered as representing the full scope of the invention.

Industrial Applicability

It will be appreciated that the present invention improves plant growth and reduces the need for fertilizers.

Claims

I claim:

1. A recombinant Rhizobium bacteria that is capable of assisting in the formation of nitrogen fixation nodules on at least some plants, the bacteria having a foreign sequence in the bacterial genome expressibly coding for trifolitoxin production.

2. The bacteria of claim 1, wherein the foreign sequence also comprises a sequence coding for resistance to trifolitoxin.

3. A plant seed inoculated with the recombinant Rhizobium bacteria of claim 1.

4. The plant seed of claim 3, wherein the foreign sequence of the recombinant bacteria also comprises a sequence coding for resistance to trifolitoxin.

5. An inoculant for a plant comprising:
a carrier; and
the recombinant Rhizobium bacteria of claim 1.

6. The inoculant of claim 5, wherein the foreign sequence of the bacteria further comprises a sequence coding for resistance to trifolitoxin.

7. A recombinant host having a foreign sequence in the host's g nome expressibly coding for trifolitoxin.

8. A recombinant Rhizobium bacteria that is capable of assisting in the formation of nitrogen fixation nodules in at least some plants, the bacteria having a foreign sequence in the bacterial genome coding for resistance to trifolitoxin.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/03109

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/31, C 12 N 1/21, A 01 C 1/06, A 01 N 63/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 01 C, A 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proc. Natl. Acad. Sci. USA, volume 85, June 1988, E.W. Triplett: "Isolation of genes involved in nodulation competitiveness from Rhizobium leguminosarum bv. trifolii T24", pages 3810-3814 see page 3810, right-hand column and page 3814, left-hand column, paragraph 5	1,2,7,8
Y	--	3-6
Y	EP, A, 0263553 (SHELL) 13 April 1988 see page 2, lines 8-14; page 3, lines 21-29; page 6, lines 7-55; page 7, lines 33-36; claims 8,19	3-6
	--	
A	Plant Molecular Biology, volume 9, no. 1, 1987, Martinus Nijhoff Publishers, (Dordrecht, NL), G. Acuna et al.: "A vector for the site-directed, genomic integration of ./.	1,2,7,8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th September 1990	17. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. Peis M. PEIS

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	foreign DNA into soybean root-nodule bacteria", pages 41-50 see the whole article, particularly page 42, left-hand column, line 7 - right-hand column	
A	Chemical Abstracts, volume 105, 1986, (Columbus, Ohio, US), D. Borthakur et al.: "A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by Rhizobium leguminosarum but not of beans by R. phaseoli and is corrected by cloned DNA from Rhizobium or the phytopathogen Xanthomonas", see page 165, abstract 92239x, & MGG, Mol. Gen. Genet. 1986, 203(2), 320-3	1,2,7,8
A	EP, A, 0245931 (LUBRIZOL) 19 November 1987 see page 3, lines 24-35; page 4, lines 40-46; page 17, lines 8-47	3-6
A	EP, A, 0242474 (AGRACETUS) 28 October 1987 see page 6, lines 10-38	3-6
A	Biological Abstracts, volume 83, no. 12, 1987, (Philadelphia, PA, US), P.M. Muriana et al.: "Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in Lactobacillus acidophilus 88", see page AB-388, abstract 117524, & Appl. Environ. Microbiol. 53(3): 553-560, 1987	1,2
P,X	Applied and Environmental Microbiology, volume 56, no. 1, January 1990, American Society for Microbiology, E.W. Triplett: "Construction of a symbiotically effective strain of Rhizobium leguminosarum bv. trifolii with increased nodulation competitiveness", pages 98-103 see the whole article	1,2,7,8
A	Biological Abstracts, volume 85, 1988, (Philadelphia, PA, US), E.W. Tripl tt et al.: "Trifolitoxin production and nodulation are necessary for the expr ssion of ./..	1-8

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	of superior nodulation competitiveness by Rhizobium leguminosarum biovar trifolii strain T24 on clover", see pages AB-1239-AB-1240, abstract 22268, & Plant Physiol. (Bethesda) 85(2): 335-342, 1987	
O,A	Biological Abstracts, BR 37:28789, E.W. Triplett et al.: "Cloning of genes from Rhizobium leguminosarum biovar trifolii T24 responsible for the production of trifolitoxin an anti-rhizobial peptide involved in nodulation competitiveness", see abstract & 7th International Congress, Cologne, West Germany, March 13-20, 1988	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9003109 SA 37876

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 10/10/90 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0263553	13-04-88	AU-A- 7903287	14-04-88
		JP-A- 63091086	21-04-88
EP-A- 0245931	19-11-87	AU-B- 593627	15-02-90
		AU-A- 7073387	01-10-87
EP-A- 0242474	28-10-87	AU-A- 6294386	26-03-87